B 3958

ANALYSIS OF THE PATHOGENESIS OF THE PHYTOPATHOGENIC FUNGUS *SCLEROTINIA SCLEROTIORUM* BY MOLECULAR METHODS

SZTE Egyetemi Könyvtár SZEGED

PhD Theses

Made by:
Kasza Zsolt

Supervisors:
Dr. Pascale Cotton
Dr. Vágvölgyi Csaba
Dr. Michel Fevre

Université Claude Bernard Lyon I,
Laboratoire Biologie Cellulaire Fongique
and
University of Szeged
Department of Microbiology
Szeged

2003

INTRODUCTION

Sclerotinia sclerotiorum is a plant pathogenic ascomycete fungus that attacks a wide range of host species. Biochemical analyses have shown that during the interaction with its host *S. sclerotiorum* secretes a complete set of cell wall-degrading enzymes (proteases, pectinases, cellulases, hemicellulases) that macerate the host tissues and provide nutrients for mycelial growth. Among the wide array of secreted cell wall degrading enzymes, polygalacturonases (PGs) are involved in the degradation of pectins, the structural polysaccharides found in the middle lamella and the pimary cell wall of higher plants. During growth on pectic polymers, several endo- and exo-enzymes are sequentially secreted. Molecular genetics has allowed the isolation of several endoPG genes (*pg1*, *pg2*, *pg3*, *pg5*).

During the infection *S. sclerotiorum* secretes oxalic acid in infected tissues which progressively shifts the pH of the plant apoplasm to acidic values provides an ideal condition for the cell wall-degrading enzymes.

THE AIMS OF THE PRESENT STUDY

- To clone the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Sclerotinia sclerotiorum*.
- To characterize the *gpd* gene at sequence level (molecular organisation, codon usage, promoter sequence) and its expression.
- To use the the *gpd* gene as a molecular marker during plant pathogenesis.
- To clone two new polygalacturonases (*pg6* and *pg7*) of *Sclerotinia sclerotiorum* and characterize them at sequence level.
- To characterize the expression of the polygalacturonase genes of *Sclerotinia sclerotiorum*.

NEW SCIENTIFIC RESULTS

Cloning and characterization of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Sclerotinia sclerotiorum*

The *gpd* gene of *S. sclerotiorum* was cloned from the genomic library. Analysis of the fragment subcloned revealed an ORF of 1133 bp and a promoter of 735 bp. The ORF contained two putative introns with conventional splice sites (5'-GT and 3'-PyAG). Analysis of the promoter sequence demonstrated the presence of several elements that could act as signals required for transcription initiation and regulation.

The genomic DNA encodes a protein containing 338 amino acids, with a molecular weight of 36.3 kDa. Alignment of the amino acid sequence with those of other ascomycetes indicates extensive homology throughout the GPD proteins. Only 44 of the 61 possible sense codons are used. Generally, the highly expressed genes show more marked codon bias than genes expressed at a low level.

On Southern-blot analysis, *S. sclerotiorum* was found to possess the *gpd* gene in a unique copy.

Expression of *gpd* during *in vitro* growth of the fungus

During a shift experiment, *S. sclerotiorum* was transferred from a glucose culture to fresh media containing different carbon sources (glucose, pectin, arabinose, rhamnose). Northern-blot analysis revealed that the *gpd* was differentially expressed.

These results prove that the *gpd* gene in *S. sclerotiorum* is not a constitutively expressed gene (a main housekeeping gene generally is considered as a constitutively expressed gene).

gpd as a molecular marker during plant pathogenesis

The *gpd* gene was highly expressed during the pathogenesis of four different plants (sunflower, bean, chicory, carrot). Northern analysis revealed similar expression profiles. The intensity of the *gpd* hybridization signals increased and reached a maximum when the plant tissues were completely invaded by the fungus.

As actin is frequently used as a marker of fungal growth during plant-fungus interactions, we compared the

expression profiles obtained with *gpd* and actin during sunflower pathogenesis. Since the level of expression did not allow detection of the actin transcript by Northern-blot, the analysis was performed by RT-PCR. The analysis disclosed similar profiles for the two genes

The results indicate that, even if *gpd* is not a constitutively expressed gene, its profile during pathogenesis reflects the amount of fungus during the interaction of *S. sclerotiorum* with its plant host. Our results prove that *gpd* can be used as a molecular (internal) marker during plant infection in order to estimate the progress of the infection and to quantify the fungal gene expression in *planta* in relation to the fungal biomass. It was confirmed that, in certain *in vitro* conditions *gpd* is a strongly expressed gene, and its promoter sequence can be used for plasmid construction for transformation.

Cloning and characterization of the endopolygalacturonases (*pg6* and *pg7*) gene of *Sclerotinia sclerotiorum*

The *pg6* and *pg7* genes were obtained by PCR amplification of *Botrytis cinerea* DNA with primers

derived from the published sequences of *Bcpg3* and *Bcpg6*. Fragments of 312 and 684 bp were amplified for *Bcpg6* and *Bcpg7*, respectively. They were then sequenced and used to screen the *S. sclerotiorum* EMBL3 genomic library.

After subcloning the sequence analysis identified 1110 bp coding sequence, 607 bp promoter and 270 bp terminator sequences for the *pg6* gene, and 1179 bp coding sequence, 549 bp promoter and 224 bp terminator sequences for the *pg7* gene. We identified 2 introns in the *pg6* and 1 intron in the *pg7* gene with the typical fungal intron sequences. Analysis of the protein sequences revealed the typical polygalacturonase motifs. Promoter sequence analysis confirmed the presence of several motifs required for the binding of transcription factors.

Functional analysis of endopolygalacturonases of *Sclerotinia sclerotiorum*

During a shift experiment, the fungus was transferred from a glucose culture to fresh media containing different carbon sources. We used a RT-PCR method (a more sensitive molecular method than Northern-blot analysis) in

order to characterize the *in vitro* expression of the *pg6* and *pg7* genes. During our *in vitro* analysis we detected only the *pg7* expression (in the presence of pectin and glucose).

In the course of the infection of carrot we examined the expressions and the roles of the four known endopolygalacturonases of *S. sclerotiorum* (*pg 2* and *pg 5* were cloned earlier and *pg 6* and *pg7* were cloned during this work). The expressions of the endopolygalacturonase genes were determined in time (0-96 h) and "in space" (five adjacent zones of carrot tissue) were sampled, each reflecting a different *Sclerotinia* infection and a different pH as a result of the oxalic acid production of *S. sclerotiorum* (pH range 3.1-6.3).

Our results revealed that *in planta* all the endopolygalacturonases (*pg2*, *pg5*, *pg6* and *pg7*) were expressed. The expression of these genes appears to be sequential in time. The sequential expression was confirmed spatially too.

We hypothesize that the oligogalacturonides generated by the individual enzymes may serve as substrates for the other endopolygalacturonase(s), like a cascade. This hypothesis can explain the results we obtained *in vitro*, when we could not find ideal (specific

vegetal) conditions to activate the expressions of *pg6* and *pg7*. The pH probably plays an essential role in the regulation of endopolygalacturonases, as indicated in our spatial experiment.

PUBLICATIONS

Vastag, M., Papp, T., **Kasza, Zs.**, and Vágvölgyi, Cs. (1998) Differentiation of *Rhizomucor* species by means of carbon source utilization and isoenzyme analysis. - J. Clin. Microbiol. 36, 2153-2156.

Vastag, M., Papp, T., **Kasza, Zs**. and Vágvölgyi, Cs. (2000) Intraspecific variation in two species of *Rhizomucor* assessed by random amplified polymorphic DNA analysis. - J. Basic Microbiol. 40, 269-277.

Vágvölgyi, Cs., **Kasza, Zs.**, Nyilasi, I., Ács, K., and Papp, T. (2001). Variability of isozyme and RAPD markers among isolates of *Mucor genevensis*. - Acta Biol. Hung. 52, 365-373.

Ács, K., **Kasza, Zs.**, Lukács, Gy., Schwab, H. and Vágvölgyi, Cs. (2002) Cloning and sequence analysis of *Mucor circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene. - Acta Microbiol. Immunol. Hung. 49, 305-312.

Kasza Zs., Cotton P., Bruel C., Rascle C., Fevre M. (2003) Ambient pH contols the expression of endopolygalacturonase genes in the necrotrophic fungus *Sclerotinia sclerotiorum*.- FEMS Microbiology Lettres (accepted)

Kasza Zs., Vágvölgyi, Cs., Fevre M, Cotton P. (2003) Molecular characterization and *in planta* detection of *Sclerotinia sclerotiorum* endopolygalacturonase genes - Current Microbiology (accepted)

Kasza Zs., Cotton P. and Fevre M. (2003) Glyceraldehyde-3-phosphate dehydrogenase as a marker of fungal growth during the interaction of the necrotrophic fungus *Sclerotinia sclerotiorum* with its plant hosts.(in preparation)

POSTER PRESENTATIONS

Vágvölgyi, Cs. Papp, T., **Kasza, Zs** (1998)- IMC6 Jerusalem –Intraspecific variability of *Mucor genevensis* assessed by isoenzyme analysis and random amplification of polymorphic DNA

Ács. K., **Kasza, Zs**., Vastag, M. and Vágvölgyi, Cs. (2000) Cloning and sequence analysis of *Mucor circinelloides* Glyceraldehyde-3-phosphate dehydrogenase gene. MMT 2000. évi Nagygyûlése, (Meeting of the Hungarian Society for Microbiology) Keszthely, Abstracts.

Ács, K., Vastag, M., **Kasza, Zs.,** and Vágvölgyi, Cs. (2001) Cloning of *Rhizomucor miehei* glyceraldehyde-3-phosphate dehydrogenase gene. MMT 2001. évi Nagygyûlése, Balatonfüred, Abstracts.

Lukács, Gy., Ács, K., Vastag, M., Nyilasi, I., **Kasza, Zs.** and Vágvölgyi, Cs. (2002) Cloning and partial sequence analysis of the gene encoding 3-hydroxy-3-methilglutharyl

coenzyme A reductase of *Rhizomucor miehei*.(2002) 3 rd Joint Meeting of the Hungarian Society for Microbiology, Balatonfüred, Hungary. Abstracts.

Vágvölgyi Cs, Vastag, M., **Kasza, Zs.,** Ács, K., (2002) Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene from *Rhizomucor miehei*. IMC7, Oslo, Abstracts

Kasza Zs, Cotton P., Vágvölgyi Cs, Fevre M. Glyceraldehyde-3-phosphate dehydrogenase as a marker of fungal growth during the interaction of the necrotrophic fungus *Sclerotinia sclerotiorum* with its plant hosts (2003) 1st FEMS Congress, Ljubjana (Slovenia)- Abstracts-with this work I won the congress junior attendance grant